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LC–MS/MS determination of betamethasone and its phosphate and acetate esters in human plasma after sample stabilization

Isam I. Salem*, Musab Alkhatib, Naji Najib

International Pharmaceutical Research Center (IPRC), Amman, Jordan

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ABSTRACT

Two specific liquid chromatography–mass spectrometric (LC–MS/MS) assays were developed and validated for the determination of betamethasone (BET), and its acetate (BA) and phosphate (BP) esters. The plasma and the blood used for the development and validation of these two methods were previously stabilized. Liquid–liquid extraction techniques were used after the addition of prednisolone as internal standard (IS). Samples were chromatographed using C8 column, while mass detection was carried out by electrospray ionization in the positive mode (ESI+). The method was proved linear over a working range 0.50–50.00 ng/ml for BET ($r^2 > 0.99$), while BA linear range was 1.0–20.0 ng/ml ($r^2 > 0.99$). Sensitivity was determined as 0.50 ng/ml for BET and 1.00 ng/ml for BA. Betamethasone phosphate LC–MS/MS method involved solid phase extraction after the addition of prednisolone phosphate as (IS). Separation was carried out using C18 column, while detection was by ESI+. The method showed good linearity over the working range 2.0–200.0 ng/ml ($r^2 > 099$). Both methods were applied to determine BET, BA and BP in plasma samples obtained for pharmacokinetics studies in human.

1. Introduction

Betamethasone, 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione (Fig. 1), is a synthetic glucocorticoid. It is active in replacement therapy for adrenal insufficiency and as an anti-inflammatory and immunosuppressant. Betamethasone is used to treat many conditions including dermatitis, arthritis, inflammatory bowel disease, reactive airways disease, and respiratory distress syndrome in preterm infants and pruritus in corticosteroid-responsive dermatoses. Betamethasone is formed by hydrolysis of the phosphate or acetate esters after intravenous or intramuscular administration to human. Other esters and salts of betamethasone are available for other routes of administration or applications, e.g., valerate, butyrate, propionate benzoate.

There are several approved products formulated based on a fast releasing betamethasone phosphate ester or as a dual acting suspension formulation containing BP and BA esters [1,2]. Both esters are expected to be hydrolyzed in vivo to the active glucocorticoid BET [3].

Several analytical techniques have been published for the analysis of BET in different matrices, e.g., high performance liquid chromatography (HPLC) [3–5], gas chromatography with mass detection (GC–MS) [6,7] or liquid chromatography with mass detection (LC–MS/MS) [8–19]. The mentioned methods showed low sensitivity, e.g., 10, 50 and 300 ng/ml [3–5], implicated derivatization [6], were not fully validated or were not applicable to clinical or pharmacokinetic (PK) studies in human [12–19]. Nevertheless, some of these methods were applied to PK studies in animals, e.g., Samtani et al. [13] reported the use of LC–MS/MS for BET determination in sheep. Though the report did not include method's validation, yet was applied to PK determination in animals after IM dose of BP/BA suspension. The authors reported BET PK, while BP and PA were not measured. The authors suggested further PK studies are needed in human.

Surprisingly, though of its major importance, to date, BET, BA and BP pharmacokinetics in human after IM administration still is not well documented in the literature. One of the reasons is due to the absence of well-documented specific and sensitive validated methods of analysis. Therefore, the main aim of our work was to develop and validate highly specific and sensitive liquid chromatographic–mass spectrometric methods for the determination of BET, BA, and BP in human plasma. These methods were to be applied for PK determination in human after IM administration of BP/BA suspension. The first method was designed for BET and BA determination. The targeted working range for BET was 0.50–50.00 ng/ml, while for BA was 1.0–20.0 ng/ml. A second method was designed to determine BP in human plasma covering the linearity over the working range of 2.0–200.0 ng/ml.

* Corresponding author at: International Pharmaceutical Research Center (IPRC), Sports City Circle, P.O. Box 963166, Amman 11196, Jordan. Tel.: +962 6 562 7651/2; fax: +962 6 562 7654.
E-mail address: dr.salem@iprc.com.jo (I.I. Salem).

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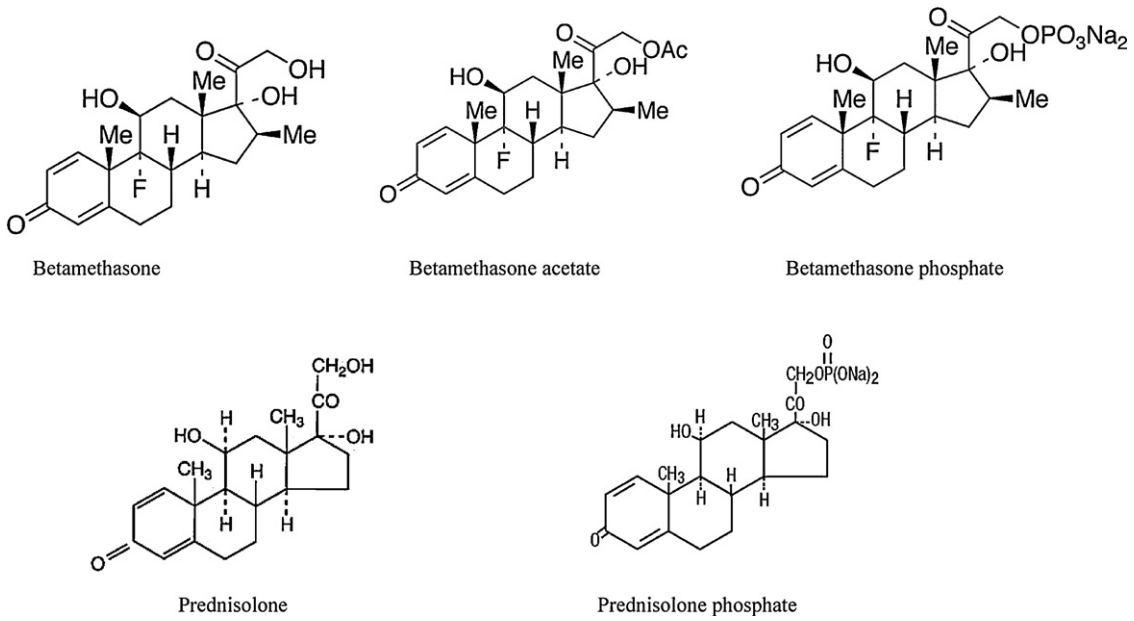

Fig. 1. Structures of BET, BA, BP and the internal standards prednisolone and prednisolone phosphate.

Furthermore, it was aim of our work to evaluate and validate arsenate and fluoride efficacy inhibiting the hydrolysis of BA and BP esters to BET in blood and plasma [3]. The methods were applied to determine the concentrations of the three analytes in the plasma obtained from healthy subjects participating in PK studies.

2. Experimental

2.1. Reagents

Betamethasone, its acetate and phosphate esters, prednisolone and its phosphate ester were obtained from TRC (Toronto, Canada). Analytical grade for HPLC acetonitrile, methanol, formic acid and diethyl ether were supplied by Merck (Darmstadt, Germany). Sodium arsenate dibasic heptahydrate, potassium fluoride anhydrous, ammonium formate, ammonium hydroxide were obtained from Sigma (UK). MCX solid phase cartridges we supplied by Waters (Milford, USA). Water was purified using a Milli-Q® (Millipore, France). Six different batches of lithium heparin blood were obtained from healthy blood donors who were proved to be HIV, hepatitis B & C negative.

2.2. Plasma pretreatment

In order to prevent the in vitro hydrolysis of BP or BA esters to BET during blood collection and plasma sample handling, the blood samples were collected into pre-chilled plastic heparinized tubes containing 10 μl 2 M sodium arsenate solution per ml blood [3]. Blood samples (kept over ice) were harvested to plasma within a maximum of 15 min by centrifugation for 5 min at 1789 × *g* and 5 °C. Plasma was siphoned into pre-chilled plastic tubes containing 10 μl of 50% (w/v) potassium fluoride solution per ml plasma [3]. Plasma samples were immediately frozen and stored at −70 °C.

2.3. Preparation of stock solutions

Stock solution of BET and BA were prepared using equivalent amounts of 10.0 mg of BET or BA in 100 ml methanol to produce a concentration of 100.0 μg/ml of either analytes. Stock solutions were stored at −20 °C. Working solutions of BET or BA were prepared by diluting in methanol to a final concentration of 10.0 μg/ml and stored at −20 °C. Serial solution of both working solutions were prepared in methanol at 10.0, 20.0 40.0, 100.0, 200.0, 400.0, 600.0 and 1000.0 ng/ml of BET, and 20.0, 40.0, 80.0, 120.0, 160.0, 240.0, 320.0 and 400.0 ng/ml of BA.

Quality control (QC) serial solutions were prepared in methanol at 10.0, 30.0, 500.0 and 840 ng/ml of BET and 20.0, 60.0, 200.0 and 340.0 ng/ml of BA.

Prednisolone (IS) working solution was prepared in methanol at 150 ng/ml and stored at −20 °C.

Stock (100.0 μg/ml) and working solution (20.0 μg/ml) for BP were prepared in methanol. BP serial dilution of 40.0, 80.0, 200.0, 400.0, 1200.0, 2400.0, 3400.0 and 4000.0 ng/ml was also prepared in methanol. Quality control samples were prepared at 40.0, 120.0, 2000.0 and 3200.0 ng/ml. The IS (prednisolone phosphate) working solution was prepared with a final concentration of 500 ng/ml. All solutions were stored at −20 °C.

2.4. Calibration curves

Calibration curve standards were prepared by spiking 50 μl of each one of the abovementioned working solutions in 1.0 ml of stabilized plasma to produce the calibration curve standards equivalent to 0.50, 1.00, 2.00, 5.00, 10.00, 20.00, 30.00 and 50.00 ng/ml of BET and 1.00, 2.00, 4.00, 6.00, 8.00, 12.00, 16.00 and 20.00 ng/ml of BA.

A double blank plasma sample (no IS) and a single blank plasma prepared containing 30.0 ng/ml of IS were used as part of each run. Neither the double blank sample nor the single blank was used to construct the calibration function. Calibration curves were run daily together with quality control samples.

Betamethasone phosphate calibration curve standards were prepared by spiking 1.0 ml stabilized plasma with 50 μl of each one of the abovementioned working solutions, producing the calibration curve standards equivalent to 2.0, 4.0, 10.0, 20.0, 60.0, 120.0, 170.0 and 200.0 ng/ml. As in the above case of BET and BA, double and single blank samples were used. The single blank contained 100.0 ng/ml of IS. Calibration curves were run daily together with quality control samples.